Materials Science and Engineering C 63 (2016) 1–9

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

The effects of crosslinkers on physical, mechanical, and cytotoxic properties of gelatin sponge prepared *via in-situ* gas foaming method as a tissue engineering scaffold

S. Ali Poursamar [a], Alexander N. Lehner [b], Mahmoud Azami [c], Somayeh Ebrahimi-Barough [c], Ali Samadikuchaksaraei [d,e,f], A.P.M. Antunes [a,*]

[a] Institute for Creative Leather Technologies, Park Campus, The University of Northampton, Boughton Green Road, Northampton NN2 7AL, UK
[b] Centre for Physical Activity and Chronic Disease and the Aging Research Centre, Institute for Health and Wellbeing, School of Health, Park Campus, The University of Northampton, Boughton Green Road, Northampton NN2 7AL, UK
[c] Department of Tissue Engineering, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
[d] Cellular and Molecular Research Centre, Iran University of Medical Sciences, Tehran, Iran
[e] Department of Tissue Engineering & Regenerative Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran
[f] Department of Medical Biotechnology, Faculty of Applied Medicine, Iran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article history:
Received 10 August 2015
Received in revised form 23 January 2016
Accepted 10 February 2016
Available online 13 February 2016

Keywords:
Gas foaming
Denaturation temperature
Over-crosslinking
Natural crosslinker

ABSTRACT

In this study porous gelatin scaffolds were prepared using *in-situ* gas foaming, and four crosslinking agents were used to determine a biocompatible and effective crosslinker that is suitable for such a method. Crosslinkers used in this study included: hexamethylene diisocyanate (HMDI), poly(ethylene glycol) diglycidyl ether (epoxy), glutaraldehyde (GTA), and genipin. The prepared porous structures were analyzed using Fourier Transform Infrared Spectroscopy (FT-IR), thermal and mechanical analysis as well as water absorption analysis. The microstructures of the prepared samples were analyzed using Scanning Electron Microscopy (SEM). The effects of the crosslinking agents were studied on the cytotoxicity of the porous structure indirectly using MTT analysis. The affinity of L929 mouse fibroblast cells for attachment on the scaffold surfaces was investigated by direct cell seeding and DAPI-staining technique. It was shown that while all of the studied crosslinking agents were capable of stabilizing prepared gelatin scaffolds, there are noticeable differences among physical and mechanical properties of samples based on the crosslinker type. Epoxy-crosslinked scaffolds showed a higher capacity for water absorption and more uniform microstructures than the rest of crosslinked samples, whereas genipin and GTA-crosslinked scaffolds demonstrated higher mechanical strength. Cytotoxicity analysis showed the superior biocompatibility of the naturally occurring genipin in comparison with other synthetic crosslinking agents, in particular relative to GTA-crosslinked samples.

1. Introduction

Gelatin has a remarkable foaming ability and can therefore be processed using gas foaming into porous structures without requiring additional surfactants and foaming agents [1]. The *in-situ* gas foaming method is a simple, non-expensive, and effective variant of the gas foaming technique [2]. It was shown that crosslinking is an important element for successful manufacturing of gelatin scaffolds *via in-situ* gas foaming and is critical in preserving the porous structure of fabricated scaffolds [3]. Different crosslinking agents have been used to stabilize gelatin tissue engineering scaffolds, four of which are the focus of this study; GTA, hexamethylene diisocyanate (HMDI), and poly(ethylene glycol) diglycidyl ether (epoxy). Fig. 1 shows the molecular structures of crosslinking agents used in this study. GTA is one of the most reported crosslinking agents in the biomedical field owing to its demonstrated effectiveness, despite being known to elicit cytotoxicity [4,5]. GTA has been applied for processing certain xenograft implants such as bioprosthetic heart valves or surgical sealants [6,7]. The GTA reaction mechanism is described as a Schiff base reaction. As a part of Schiff base reactions, carbon and nitrogen double bonds (C=N) are established between GTA and the amine groups of Lysine in gelatin molecules [8].

As an alternative to synthetic crosslinkers such as GTA naturally occurring crosslinking agents such as genipin have been used. Genipin is extracted from the fruits of plants that are native to South America and South East Asia and has been used in Chinese herbal medicine [9]. With less toxicity relative to GTA, genipin reacts with amino-containing materials and has been used in crosslinking gelatin microcapsules for drug delivery, conduits for peripheral nerve regeneration, and composites for Guided Bone Regeneration [10].

* Corresponding author. Tel.: +44 1604 892543; fax: +44 1604 711183.
E-mail address: Paula.Antunes@northampton.ac.uk (A.P.M. Antunes).

http://dx.doi.org/10.1016/j.msec.2016.02.034
0928-4931/

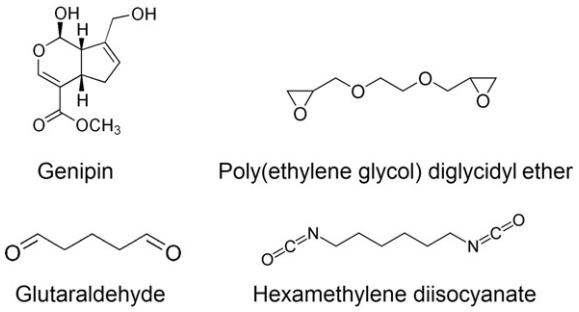

Fig. 1. Molecular structures of crosslinkers used in this study.

HMDI molecules with two cyanate groups react either with amine groups or hydroxyl groups of gelatin to form urea or urethane bonds, respectively. HMDI has been reported as a crosslinking agent for applications in bone and cartilage tissue engineering [11], and as a coupling agent for the surface treatment of metallic and ceramic materials [12,13].

The driving force behind the crosslinking reaction that involves epoxy molecules is the strained covalent bonds that compose three-atom-ether rings at both ends of poly(ethylene glycol) diglycidyl ether. One practical advantage of epoxy compounds is their ability in reacting with a variety of functional groups (this includes carboxylic, amine, and hydroxyl groups) at a wide range of pH's [14]. Fig. 2 summarizes the reaction mechanisms of applied crosslinking agents.

In our previous study the application of crosslinking agents proved to be an essential part of *in-situ* gas foaming [3]. Although crosslinking has a deep impact on the mechanical and physical properties of samples prepared *via in-situ* gas foaming, there is no comprehensive study that identifies suitable crosslinking agent for application in this method, to the best of the authors' knowledge. In addition this study strives to add to the relatively limited number of reports on HMDI application as a crosslinker for gelatin samples.

Here, a comparative study is reported with the aim of identifying a biocompatible and effective crosslinker for application in *in-situ* gas foaming. The properties of prepared samples were characterized using a Scanning Electron Microscope (SEM), Fourier Transform Infrared spectroscopy (FT-IR), thermal analysis, tensile strength analysis, and water absorption analysis. To compare the biocompatibility of the samples L929 fibroblast cells were cultured, stained, and fixed to study their spreading and attachment using confocal and scanning electron microscopes.

2. Materials and methods

2.1. Scaffold preparation method

Porous gelatin scaffolds were prepared according to the method described earlier with some modifications [3]. Briefly, type B gelatin powder (Sigma Aldrich, USA) was used to prepare a 20% w/v aqueous solution. Sodium bicarbonate (BDH Chemical, UK) was directly added to the gelatin solution. Consequently, a 360 μl aliquot of acetic acid (Fisher Scientific, UK) was added to initiate the foaming process. The gelatin foam was cast in the polystyrene molds, 5.5 cm in diameter, and frozen for 1 h at −25 °C. The frozen foam blocks were then extracted from the molds and incubated in 4 °C de-ionized water to extract unreacted components. The preparation process was continued with the samples crosslinking as follows:

Crosslinking methods:

GTA Crosslinking: Upon removal from 4 °C de-ionized water, the samples were incubated in aqueous solutions of GTA for 3 h. GTA aqueous solutions were prepared from a 50% *v*/v aqueous stock solution of GTA (Fisher Scientific, UK). An aliquot of 500 μl GTA was added to 50 ml de-ionized water providing 0.5 mol/v GTA solution.

Epoxy Crosslinking: An aliquot of 1.15 ml poly(ethylene glycol) diglycidyl (Sigma Life Science, Japan) was added to 50 ml of de-ionized water making 0.5 mol/v solution. Upon removal from 4 °C de-ionized water, the samples were incubated in the prepared epoxy solution for 3 h.

HMDI Crosslinking: Dehydration of samples and crosslinking of scaffolds with HMDI was performed according to the method reported by Catalina et al. (2011). The samples were dehydrated in a gradient of propan-2-ol aqueous solutions of 25, 50, 75, and 100% v/v (each step 30 min under agitation). Consequently, the scaffolds were incubated in propan-2-ol solution of HMDI at 4 °C for 16 h. To prepare the HMDI solution, 400 μl of HMDI was added to 50 ml of propon-2-ol giving 0.5 mol/v concentration. After crosslinking, the samples were rehydrated in a gradient of propan-2-ol aqueous solutions in the reverse order used in pre-crosslinking preparation (*i.e.* 100, 75, 50, 25% v/v propan-2-ol aqueous solutions).

Genipin Crosslinking: The crosslinking of samples was performed according to the method reported by Bigi et al. [15]. Soaked samples were incubated in Phosphate Buffered Solution (PBS) of genipin for 14 h at room temperature without agitation. To prepare the crosslinking solution, 1.13 g of genipin (Challenge Bioproducts, Taiwan) was added to 100 ml of PBS solution providing a genipin solution with concentration of 0.5 mol/v.

Upon crosslinking, the samples were washed in de-ionized water overnight, frozen, and lyophilized at −40 °C and under a vacuum pressure of 0.250 mbar for 1 day. Non-crosslinked samples were prepared for comparison by removing the crosslinking step from the process.

2.2. Characterization of the prepared scaffolds

2.2.1. Mechanical properties

Once the samples were conditioned at 95% relative humidity for 2 days, the mechanical testing was carried out using a texture analyzer (TA.XT-Plus, Stable Micro Systems, UK). Samples were cut into rectangular strips (10 × 5 mm), thicknesses were measured at 3 points, and the average value was recorded. The samples were drawn at a cross head speed of 0.033 $mm \cdot s^{-1}$. The tensile strength and strain values are reported in kPa and percentage (%), respectively. The Young's modulus of the scaffolds was calculated as the slope of the linear segment of Stress–Strain curve and is reported in kPa. The tests were performed in triplicate.

2.2.2. Thermal analysis

The samples were conditioned at 65% relative humidity for 2 days prior to analysis. Differential Scanning Calorimetry (DSC — 822e, Mettler-Toledo, Switzerland) was used for thermal analysis. The samples were sealed in 40 μl aluminum pans and heated from 15 to 100 °C at a heating rate of 5 $°C \cdot min^{-1}$. The peak temperature and the normalized enthalpy of transition of each sample were recorded. The peak temperature was assigned as the gelatin denaturation temperature (T_d). The experiments were performed in triplicate.

2.2.3. Fourier Transform Infra-Red Spectroscopy (FT-IR)

Fourier Transform Infra-Red Spectroscopy (FTIR/ATR-4800s, Shimadzu, Japan) was performed by scanning from 4000 to 1000 cm^{-1} at a nominal resolution of 4 cm^{-1} using 264 scans. Multiple scans were performed on each sample and a representative FT-IR diagram for each sample is chosen for presentation.

2.2.4. Swelling ratio

The samples were conditioned in a 0% relative humidity desiccator for 2 days. Dry samples were weighed and incubated in de-ionized water. To prevent non-crosslinked sample disintegration in water during analysis, the samples were kept at 4 °C (below gelling point of gelatin). The hydrated samples were removed from water at intervals of 1, 3, and 6 h. Removal of excess superficial water with filter paper was carried out and the sample weights were recorded. Measurement for each batch of samples was carried out in duplicate and the average value of the two results is reported. The swelling ratio was calculated